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FULL-LENGTH ORIGINAL RESEARCH

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Phenobarbital and midazolam suppress neonatal seizures in a noninvasive rat model of birth asphyxia, whereas bumetanide is ineffective

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Abstract

Objective: Neonatal seizures are the most frequent type of neurological emergency in newborn infants, often being a consequence of prolonged perinatal asphyxia. Phenobarbital is currently the most widely used antiseizure drug for treatment of neonatal seizures, but fails to stop them in ~50% of cases. In a neonatal hypoxia-only model based on 11-day-old (P11) rats, the NKCC1 inhibitor bumetanide was reported to potentiate the antiseizure activity of phenobarbital, whereas it was ineffective in a human trial in neonates. The aim of this study was to evaluate the effect of clinically relevant doses of bumetanide as add-on to phenobarbital on neonatal seizures in a noninvasive model of birth asphyxia in P11 rats, designed for better translation to the human term neonate.

Methods: Intermittent asphyxia was induced for 30 minutes by exposing the rat pups to three 7 + 3-minute cycles of 9% and 5% O₂ at constant 20% CO₂. Drug treatments were administered intraperitoneally either before or immediately after asphyxia.

Results: All untreated rat pups had seizures within 10 minutes after termination of asphyxia. Phenobarbital significantly blocked seizures when applied before asphyxia at 30 mg/kg but not 15 mg/kg. Administration of phenobarbital after asphyxia was ineffective, whereas midazolam (0.3 or 1 mg/kg) exerted significant antiseizure effects when administered before or after asphyxia. In general, focal seizures were more resistant to treatment than generalized convulsive seizures. Bumetanide (0.3 mg/kg) alone or in combination with phenobarbital (15 or 30 mg/kg) exerted no significant effect on seizure occurrence.

Significance: The data demonstrate that bumetanide does not increase the efficacy of phenobarbital in a model of birth asphyxia, which is consistent with the negative data of the recent human trial. The translational data obtained with the novel rat model of birth asphyxia indicate that it is a useful tool to evaluate novel treatments for neonatal seizures.

KEYWORDS

antiseizure drugs, GABA, neonates, NKCC1

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1 | INTRODUCTION

Neonatal seizures are the most common neurological emergency in the neonatal period, occurring in 1-5 per 1000 live births.¹ Prolonged perinatal asphyxia is a major cause of seizures and hypoxic-ischemic encephalopathy in the neonatal population.¹⁻³ Notwithstanding its potential toxicity in neonates,⁴ the antiseizure drug (ASD) phenobarbital is still the first-line therapy. However, despite the use of high doses of phenobarbital (10-40 mg/kg), seizures are controlled in fewer than one-half of the neonates.^{2,5} Variable efficacy (ranging from minimal to 100%) has been reported for the benzodiazepine, midazolam, as a second-line medication.² Neonates with continued seizures have a poor prognosis, including significant mortality and, in survivors, serious sequelae such as mental retardation, a wide range of neuropsychiatric disorders, motor deficits, and epilepsy.² Thus, improving the treatment of neonatal seizures is an urgent medical need.^{5,6}

Phenobarbital potentiates the effect of the inhibitory neurotransmitter γ -aminobutyric acid (GABA) by enhancing the hyperpolarizing chloride (Cl^-) ion-mediated current across GABA_A receptors in target neurons.⁷ The transmembrane electrochemical Cl^- gradient of neurons is set by the interaction of Cl^- -permeable channels and by secondary-active ion-transporters, of which the Cl^- importer NKCC1 and the Cl^- exporter KCC2 are the major ones.⁸ Several studies have shown that KCC2 expression in the human neonate is high,⁸ which results in the low intracellular Cl^- concentration required for the hyperpolarizing action of GABA. However, following neuronal trauma, downregulation KCC2 and upregulation of NKCC1 may occur. As a result, Cl^- inbound transport exceeds the outbound transport, so that intracellular Cl^- levels are high, thus resulting in a reversed Cl^- gradient. This results in a change in the functionality of GABA in damaged neurons, which becomes depolarizing/excitatory rather than hyperpolarizing/inhibitory, possibly explaining the poor efficacy of phenobarbital and other ASDs (eg, benzodiazepines such as midazolam) that act via GABAergic mechanisms in neonates.⁹ However, whether and to what extent this mechanistic scheme is valid for the brain of the asphyxiated human neonate is not known.

Based on preclinical studies, the potent loop diuretic, bumetanide, has been proposed to be useful in suppressing seizures following perinatal brain injury, because it may restore GABAergic inhibition by blocking neuronal NKCC1 in the brain, thereby increasing the efficacy of phenobarbital and other positive modulators of GABA_A receptors to suppress neonatal seizures.¹⁰⁻¹² This prompted two clinical trials in newborn babies with seizures to examine the efficacy of bumetanide, administered together with phenobarbital (US Food and Drug Administration Investigational New Drug No. 101690: <http://clinicaltrials.gov/ct2/show/NCT00>

Key Points

- Neonatal seizures are the most common neurological emergency in the neonatal period and only poorly respond to antiseizure drugs
- Neonates with continued seizures have a poor prognosis, so improving the treatment of neonatal seizures is an urgent medical need
- In rodents, bumetanide was reported to potentiate the antiseizure activity of phenobarbital, but was ineffective in a clinical trial in neonates
- We used a novel rat model of birth asphyxia and found that phenobarbital and midazolam partially suppressed the seizures, whereas bumetanide was ineffective
- These data indicate that this model is a useful tool to evaluate novel treatments for neonatal seizures

830531; EU FP7 NEMO: <http://www.nemo-europe.com/>). The European trial with bumetanide (0.05-0.3 mg/kg) as adjunct to phenobarbital (10 mg/kg) for treatment of neonatal seizures was stopped early because of serious adverse reactions (ototoxicity) and no evidence for seizure reduction.¹³ The US trial was started in 2009, but the results have not been published as yet.

The negative outcome of the European trial raises questions about the translational value of the preclinical models that have been used to evaluate the effect of bumetanide and phenobarbital combinations. For instance, in the study of Cleary et al,¹⁴ 10-day-old (P10) rat pups were exposed to hypoxia for 15 minutes, which induced acute seizures in 95% of vehicle controls during the hypoxia. When phenobarbital (15 mg/kg intraperitoneal [ip]) and/or bumetanide (0.15 or 0.3 mg/kg ip) were administered prior to seizure induction, phenobarbital or bumetanide alone did not significantly reduce seizure incidence, whereas a reduction by 75%-85% was obtained by combining either dose of bumetanide with phenobarbital.¹⁴ The authors concluded that “these data provide preclinical support for clinical trials of bumetanide in human neonates at risk for hypoxic encephalopathy and seizures.” However, in human asphyxiated neonates, seizures occur mainly during the recovery period following perinatal asphyxia,^{2,3} not during the hypoxic episode associated with complicated birth. Asphyxia is, by definition, a combination of hypoxia and hypercapnia, and recent work has shown the elevation in brain CO_2 levels has robust brain-sparing effects,¹⁵ whereas the recovery to normocapnia (and the associated rise in pH) promotes seizures in the model used in the present study.¹⁶

The aim of this study was to evaluate whether the anti-seizure effect of bumetanide/phenobarbital combinations reported by Cleary et al¹⁴ for a rat hypoxia-only model of neonatal seizures is also observed in a novel asphyxia model of such seizures. For this goal, we used a model of birth asphyxia seizures recently described by us in P11-12 rats,¹⁶ which correspond to term human babies.¹⁷⁻¹⁹ In contrast to hypoxia-only models such as the one used by Cleary et al,¹⁴ seizures in this asphyxia model develop after the insult,¹⁶ which closely parallels the situation in human neonates. For characterizing the pharmacology of the asphyxia-induced seizures in rat pups, we administered phenobarbital and the potent benzodiazepine, midazolam, either before or after the period of asphyxia. Furthermore, we tested bumetanide alone and in combination with phenobarbital, using the doses and administration schedule described in the study of Cleary et al.¹⁴

2 | MATERIALS AND METHODS

2.1 | Animals

Breeding pairs of Wistar Han (CrI:WI[Han] outbred) rats were obtained from Charles River. The animals were kept under controlled environmental conditions (22-24°C; 50%-60% humidity; 12-hour light/dark cycle; light on at 6:00 AM) with free access to standard laboratory chow (Altromin 1324 standard diet; Altromin) and tap water. Male and female offspring were used at P11 (where P0 refers to the day of birth). Each litter was separated into pups that served as controls and pups that were used for drug treatment as described below. The numbers of breeder pairs and litters used for the present study are shown in Table S1.

Experiments were performed in Hannover, Germany, according to the EU council directive 2010/63/EU and the German Law on Animal Protection (Tierschutzgesetz). Ethical approval for the study was granted by an ethical committee (according to §15 of the Tierschutzgesetz) and the governmental agency (Lower Saxony State Office for Consumer Protection and Food Safety) responsible for approval of animal experiments in Lower Saxony (reference number for this project: 17/2604). All efforts were made to minimize both the suffering and the number of animals. A total of 173 rat pups were used for the present experiments. All animal experiments of this study are reported in accordance with ARRIVE guidelines.²⁰

2.2 | Asphyxia model of neonatal seizures

Details of this novel model are reported elsewhere.^{15,16} For the present experiments, the intermittent asphyxia protocol

was slightly modified, because the previously used protocol with alternating 5-minute periods of 9% O₂/20% CO₂ and 5% O₂/20% CO₂ with a total duration of 30 minutes was associated with high mortality in the present experiments, most likely attributable to subtle substrain differences in the Wistar outbred rats used in the present and previous work (cited above). The protocol illustrated in Figure 1 was used. In short, the rat pups were placed in exposure chambers (one per chamber), with a constant temperature of 35.5°C, 15 min prior to exposure to asphyxia; under these conditions the animals' rectal temperature stabilizes to 36-37 °C.¹⁶ Intermittent asphyxia was induced for 30 minutes by exposing the rat pups to three cycles of 7 minutes 9% O₂ and 3 minutes 5% O₂ at a constant elevated level of CO₂ (20% of atmospheric pressure), as illustrated in Figure 1. Gases were humidified and delivered at a flow rate of 3000 mL/min into two exposure chambers; the size of each chamber was 12 × 12.2 × 12.3 cm, resulting in a chamber volume of 1800 cm³. When possible, control and drug-treated pups were performed in parallel, using one chamber per animal. After the asphyxia protocol, animals were promptly re-exposed to room air. Animals were closely observed during and for at least 10 minutes after asphyxia by two investigators, who were blinded to the treatments. Furthermore, the animals' behavior was video-monitored by two cameras, one from the side and one from above, to allow offline post hoc analyses of behavioral alterations. In the absence of drug treatment, all animals developed seizures within 10 minutes after the end of asphyxia (see Results). The type and severity of these seizures were scored by a modified Racine scale²¹ as described in Table 1. Furthermore, paroxysmal behavioral symptoms and automatisms that could not be rated by this scale were noted. Mortality associated with asphyxia was recorded. For sham asphyxia, all experimental procedures were identical, except that the pups were exposed to room air in the chambers used for asphyxia.

In some experiments, the cortical electroencephalogram (EEG) was recorded before, during and after asphyxia. Epidural EEG screw electrodes were implanted into the skull above the motor cortex 1 day before asphyxia under anesthesia with isoflurane. Stereotactic coordinates for the cortical electrode in millimeters from bregma²² were anteroposterior (AP), +1.5; mediolateral (ML), +1.5. A reference screw electrode was implanted at AP, +1.5; ML, -1.5; and a grounding screw at AP, -3.8; ML, +2.0. The EEG was recorded in freely moving rat pups with simultaneous video recording, using the parameters and EEG/video equipment described in detail previously.²³

2.3 | Drug treatment

As shown in Figure 1, three drugs were evaluated: phenobarbital (15 or 30 mg/kg ip), midazolam (0.3 or 1 mg/kg

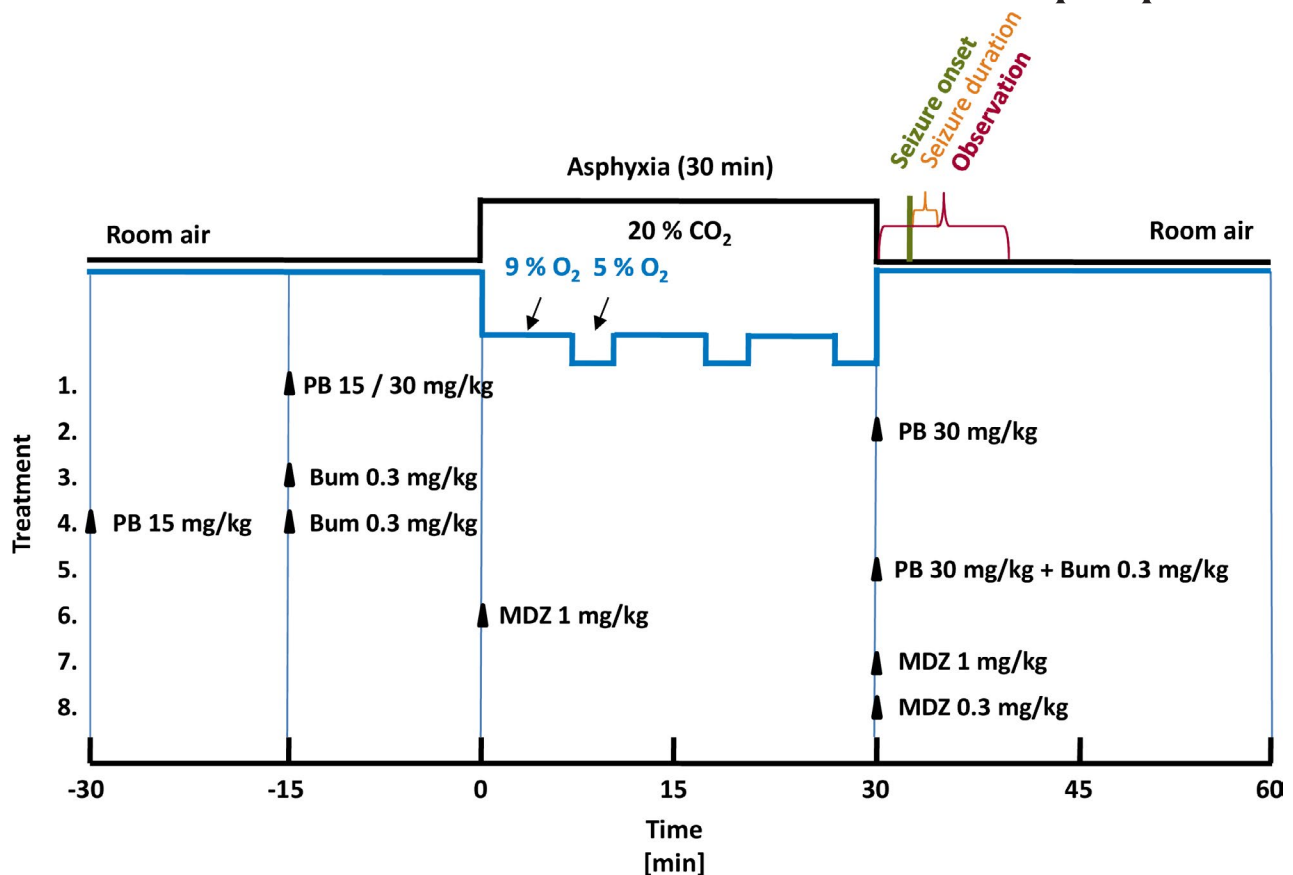


FIGURE 1 Schematic presentation of the experimental protocol used for the present experiments. Intermittent asphyxia was induced for 30 minutes by exposing the rat pups to three cycles of 7 minutes to 9% O₂ and for 3 minutes to 5% O₂ at a constant elevated level of CO₂ (20% of atmospheric pressure). After asphyxia, the average time of onset and duration of seizure activity and the average duration of observation of the rat pups are indicated. For details, see Ala-Kurikka et al.¹⁶ Bum, bumetanide; MDZ, midazolam; PB, phenobarbital

ip), and bumetanide (0.3 mg/kg ip). The latter drug was administered either alone or in combination with phenobarbital (15 mg/kg or 30 mg/kg ip). Doses of phenobarbital and bumetanide were those used by Cleary et al.¹⁴ These doses are in the range used clinically in human neonates.³ In experiments, in which phenobarbital and bumetanide were administered before asphyxia, we used the pretreatment times described by Cleary et al.¹⁴ as illustrated in Figure 1. Midazolam was injected immediately before or after asphyxia. Furthermore, in an additional experiment, phenobarbital (30 mg/kg ip) was injected 15 minutes before or immediately after asphyxia. Because phenobarbital (30 mg/kg) was not effective when administered immediately after asphyxia (see Results), we examined whether bumetanide (0.3 mg/kg) administered together with phenobarbital (30 mg/kg) after asphyxia increases the efficacy of phenobarbital. Together with each drug experiment, a group of untreated rat pups was tested in parallel in a treatment-blind manner. Rat pups were randomly assigned to the different groups. For midazolam, which was dissolved in sterile water for injection, concurrent controls received the vehicle. This also allowed evaluating whether vehicle

injection induced any differences compared to untreated controls. Maternal separation was equally long in test and control animals.

2.4 | Drug analysis in plasma and brain

Some drug-treated animals were killed 10 minutes after asphyxia (after the seizure surveillance period) for determining drug concentrations in plasma and brain. Phenobarbital, bumetanide, and midazolam were extracted from plasma and brain and determined by high-performance liquid chromatography with ultraviolet detection as described previously.^{23,24} Detection limit for bumetanide in the brain tissue was 0.009 µg/g.

2.5 | Drugs

Phenobarbital (used as sodium salt; Serva) was dissolved in sterile water. For experiments in which bumetanide (Sigma) was administered before asphyxia, it was dissolved in a

TABLE 1 Different types of neonatal seizures and seizure scoring system used for grading seizure severity in a postnatal day 11 rat asphyxia model

Seizure stage	Behavioral symptoms	Alterations in cortical EEG	Comments	Clinical correlates in neonates
I	Mouth or facial clonus	No change observed	Nonconvulsive (limbic?)	Oral-buccal automatisms (eg, mouthing, chewing)
II	Head nodding/shaking and/or ≥ 2 myoclonic twitches of whole body (rapid isolated jerks)	No change observed	Nonconvulsive (limbic?)	Epileptic spasms (may result in head nodding) and/or rapid isolated myoclonic jerks (random single contractions)
III	Uni- or bilateral repetitive clonic seizures of limbs (without loss of righting)	Epileptic activity (see Figure 2)	Generalized convulsive (motor)	Clonic seizures (repetitive jerking, unifocal or multifocal); seizure type best recognized clinically
IV	Uni- or bilateral repetitive clonic seizures of limbs and tonic seizures of fore- or hindlimbs (without loss of righting)	Epileptic activity (see Figure 2)	Generalized convulsive (motor); rearing (as in the original Racine scale) is not observed in the rat pups	Clonic seizures (repetitive jerking, unifocal or multifocal)
V	Repetitive clonic seizures of limbs and tonic seizures of fore- or hindlimbs (\pm wild running) with loss of righting (falling)	Epileptic activity (see Figure 2)	Generalized convulsive (motor)	Tonic stiffening; decerebrate posturing; usually focal, unilateral or bilateral asymmetric; sustained posturing of limb/trunk
Other paroxysmal symptoms (not rated)	Backing; Straub tail; limb posturing and movements (eg, swimming, pedaling); flexion or bending of body or limbs resembling infantile spasms	No change observed		Subtle seizures are the most common seizure type in both preterm and term babies; manifestations include ocular phenomena (staring, blinking, eye deviation, eye opening), oral phenomena (see stage I), fragmentary body movements (limb posturing, swimming, pedaling), autonomic phenomena
Stereotyped behaviors (not rated)	Grooming, circling, wet-dog shakes	No change observed	May occur before, in-between, or after seizures; not clear whether these behaviors represent nonconvulsive limbic seizures	Motor automatisms

Note. For clinically obvious seizures, seizure type and severity were graded by a modified Racine scale. The different seizure stages do not have to occur in sequence (from lowest to highest stage), but, presumably because of involvement of different brain structures, may occur in a mixed manner. Seizures originating presumably from the limbic system (eg, mouth clonus, stereotypies) are likely to occur before seizures originating from forebrain cortical areas (eg, clonic) or the brain stem (tonic seizures). None of the clinically obvious seizures or other paroxysmal behavioral symptoms shown in the table occurred after sham asphyxia (see text). For the clinically obvious seizures and other paroxysmal behavioral symptoms occurring after asphyxia in rat pups, clinical correlates in neonates are shown for comparison (based on the current International League Against Epilepsy classification recently reported by Pressler et al¹).

Abbreviation: EEG, electroencephalogram.

vehicle containing 5% ethanol, 5% Solutol HS 15 (a nonionic solubilizer consisting of ethoxylated 12-hydroxystearic acid; BASF), and 90% distilled water. For experiments in which bumetanide was administered together with phenobarbital after asphyxia, we dissolved this drug together with phenobarbital in an aqueous 10% solution of hydroxypropyl- β -cyclodextrin (HP β CD; Kleptose, Roquette-Pharma), which contained 2.25% glucose. Preliminary experiments with solutions of bumetanide in ethanol/Solutol vs HP β CD did not indicate any differences in drug pharmacokinetics, including brain distribution. The reason for avoiding the ethanol/Solutol solution shortly after asphyxia was the potential antiseizure activity of ethanol administered shortly before onset of seizures.²³ Midazolam was used as commercial aqueous solution of its hydrochloride (midazolam-hameln), which was diluted by sterile water. All drugs were injected ip; injection volume was 10 mL/kg, except for combined treatment with bumetanide and phenobarbital before asphyxia, for which injection volume was 3 mL/kg per drug, and treatment with midazolam, for which injection volume was 1.7 mL/kg.

2.6 | Statistics

In all experiments, rat pups from a given litter were randomly assigned to the drug and vehicle groups and experiments were performed in a blinded fashion. Statistical analyses were performed using GraphPad Prism 8.0 software. Either parametric or nonparametric tests were used for statistical evaluation, depending on data distribution. For comparison of two groups, either Student *t* test, Mann-Whitney *U* test, or Wilcoxon signed rank test was used, depending on whether data were paired. In case of more than two groups, we used analysis of variance (ANOVA) with post hoc testing and correction for multiple comparisons. Depending on data distribution, either the ANOVA *F* test, followed post hoc by Dunnett multiple comparisons test, or the Kruskal-Wallis test followed post hoc by Dunn multiple comparisons test was used. For comparison of frequencies in a 2 \times 2 table, Barnard unconditional test²⁵ was used, because this test preserves the significance level and generally is more powerful than Fisher exact test for moderate to small samples.²⁶ All tests were used two-sided; *P* < .05 was considered significant. G*Power Data Analysis²⁷ was used to calculate the necessary sample size for a specified power of 80%.

3 | RESULTS

3.1 | Behavioral alterations during and after asphyxia in P11 rats

Exposing the rat pups to the asphyxia protocol shown in Figure 1 did not produce any obvious behavioral effects, apart

from brief agitation on initiation of the exposure. Seizures were never seen during the asphyxia. However, following establishment of normocapnic conditions after asphyxia, all rat pups exhibited clinically obvious seizures (stage I-V) within 0.5-7.4 minutes (mean = 2.1 minutes). These seizures occurred repeatedly over a period of 0.5-8.97 minutes (mean = 3.7 minutes) postasphyxia, after which the animals resumed normal behavior. In agreement with previous findings¹⁶ and as shown in Table 2, the predominant seizure type was generalized convulsive (stage III-V) seizures, particularly generalized tonic-clonic (stage V) seizures. The average latency to convulsive stage III-V seizures was 2.2 minutes (range = 1.0-3.88 minutes). Individual group values for seizure latencies are shown in Table S2. Representative examples of seizures are shown in Video S1.

In addition, several other paroxysmal symptoms, which were not observed following sham asphyxia, were determined in the pups, including fragmentary body movements (limb posturing, swimming, pedaling) that are also seen following asphyxia in human neonates (Table 1; Video S1). Similarly, stereotyped behaviors, described as motor automatisms during human neonatal seizures, were determined in the rat pups (Table 1; Video S1). When these other paroxysmal symptoms and automatisms were included in calculation of latency to first symptom after termination of asphyxia, a latency period of 0.33-3.5 minutes (mean = 1.31 minutes) was obtained. In this respect, it is important to note that subtle seizures are the most common seizure type in both preterm and term babies, with manifestations such as ocular phenomena (staring, blinking, eye deviation, eye opening), oral phenomena, fragmentary body movements (limb posturing, swimming, pedaling), and autonomic phenomena.¹ In the rat pups of the present asphyxia model, distinct types of such subtle seizures were observed in 30%-60% of the animals (Table 2).

Again, in agreement with previous work,¹⁶ the typical generalized convulsive (stage III-V) seizures recorded after asphyxia were paralleled by paroxysmal, epileptic activity in the cortical EEG (Table 1). Examples of such EEG alterations are illustrated in Figure 2B-D. The clinical seizures observed during EEG recording are illustrated above each EEG trace. In apparent contrast to generalized convulsive stage III-V seizures, stage I/II limbic seizures and the other paroxysmal symptoms and automatisms observed in the rat pups after asphyxia were not paralleled by cortical EEG alterations (Table 1), indicating that they arose from subcortical or paleocortical areas such as the limbic system. During asphyxia, the EEG amplitude was suppressed compared to the preasphyxia baseline (Figure 2A).

The average number of clinically obvious (stage I-V) seizures following termination of asphyxia was 2.44 (range = 1-5) per animal. Seizures typically progressed through several stages (see examples in Figure 2). Average seizure severity (using the highest rated seizure per animal) was 3.83 (range = 1-5).

TABLE 2 Effect of intraperitoneal treatment with bumetanide, phenobarbital, bumetanide plus phenobarbital, or midazolam on seizures occurring after asphyxia in P11 rats

Clinically obvious seizures and other paroxysmal behavioral symptoms and stereotypies after asphyxia in P11 rat pups	Treatment before asphyxia					Treatment after asphyxia			
	Untreated, all	Vehicle, all	Phenobarbital 15mg/kg	Phenobarbital mg/kg	Phenobarbital 30	Bumetanide 0.3 mg/kg	Bumetanide 0.3 mg/kg + phenobarbital 15 mg/kg	Midazolam 1 mg/kg	Phenobarbital 30 mg/kg + Bumetanide 0.3 mg/kg
Number of pups tested	27	28	8	9	26	11	6	13	19
No seizures	0/23	0/28	4/8	6/8	1/17	2/10	4/5	0/13	5/15 (<i>P</i> = .00015)
Nonconvulsive seizures [focal; I-II]	8/23	1/28	2/8	2/8	7/17	4/10	1/5	1/13	4/19 (<i>P</i> < .0001)
Convulsive seizures [III-V]	15/23	27/28	2/8	0/8 (<i>P</i> = .011)	9/17	4/10	0/5 (<i>P</i> = .0002)	12/13	5/19 (<i>P</i> < .0001)
Total [I-V]	23/23	28/28	4/8 (<i>P</i> = .0041)	2/8 (<i>P</i> = .000012)	16/17	8/10	1/5 (<i>P</i> = .0018)	13/13	9/19 (<i>P</i> = .00015)
Average seizure severity, score ± SEM	3.83 ± 0.32	4.5 ± 0.17	1.25 ± 0.49 (<i>P</i> = .0004)	0.38 ± 0.26 (<i>P</i> < .0001)	3.18 ± 0.41	2.2 ± 0.47 (<i>P</i> = .0084)	0.2 ± 0.2 (<i>P</i> < .0001)	4.77 ± 0.22	1.53 ± 0.47 (<i>P</i> < .0001)
Other paroxysmal symptoms [see Table 1]	8/23	13/28	3/8	0/8 (<i>P</i> = .049)	7/17	3/10	0/5	2/13	5/19 (<i>P</i> = .026)
Stereotypies [see Table 1]	7/23	15/28	1/8	1/8	7/17	5/10	0/5	11/13 (<i>P</i> = .0018)	2/19 (<i>P</i> = .00064)
Total [seizures and other paroxysmal symptoms or stereotypies]	23/23	28/28	5/8 (<i>P</i> = .039)	2/8 (<i>P</i> = .000012)	16/17	10/10	1/5 (<i>P</i> = .0018)	13/13	9/19 (<i>P</i> = .00015)
Mortality	4/27	0/28	0/8	1/9	9/26	1/11	1/6	0/13	0/19

Note.: Drug treatment was either performed before asphyxia or immediately after asphyxia as shown in Figure 1. Significant differences from either untreated animals or, for midazolam, from a concurrent vehicle group are shown. Total incidence of seizures or other paroxysmal symptoms in untreated animals did not significantly differ from those observed in the vehicle group. *P* values of significant differences between drug-treated groups and controls are indicated. No sex differences were observed in any group (see text).
Abbreviation: P11, postnatal day 11.

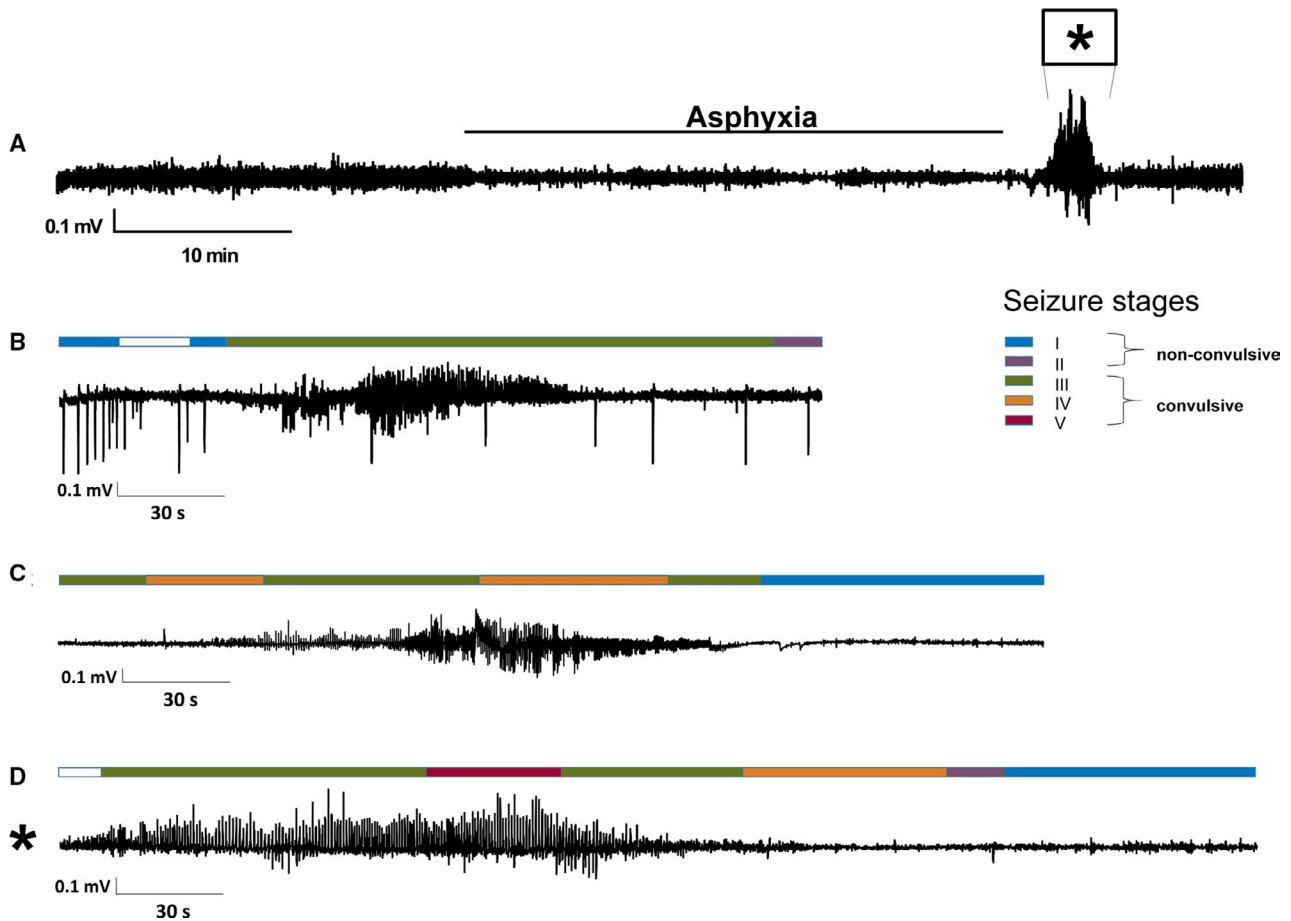


FIGURE 2 Representative examples of cortical electroencephalographic (EEG) recordings in postnatal day 11 rat pups. A, EEG before onset of asphyxia, during asphyxia, and in the recovery period after asphyxia. Note EEG suppression during asphyxia and the seizures (indicated by asterisk) occurring during the recovery phase. These seizures are shown in more detail in D below. In B–D, clinical seizures observed during EEG recording are illustrated above each EEG trace, using the colors indicated in the seizure stage legend in the figure. B, Paroxysmal EEG alterations during evolution of postasphyxic seizures from stage I (nonconvulsive) to stage III (convulsive). Note that nonconvulsive seizure activity was observed before and after convulsive seizure activity. C, Paroxysmal EEG alterations during evolution of postasphyxic seizures from stage III to stage IV. Note that nonconvulsive seizure activity was observed after convulsive seizure activity. D, Paroxysmal EEG alterations during evolution of postasphyxic seizures from stage III to stage V. Note that nonconvulsive seizure activity was observed after convulsive seizure activity

When data were calculated separately for male ($n = 14$) and female pups ($n = 9$), no significant differences in average latency to first seizure (1.5 ± 0.2 minutes vs 1.8 ± 0.4 ; $P = .4662$), the average period over which seizures were observed (3.8 ± 0.7 minutes vs 3.0 ± 0.7 minutes; $P = .4504$), and the average number of seizures (2.7 ± 0.3 vs 2.9 ± 0.4 seizures/pup; $P = .6888$) were obtained. Furthermore, as shown in Figure S1, all untreated controls and all vehicle-injected controls of both sexes exhibited 100% seizure incidence.

Rat pups that were injected with vehicle (sterile water) before or after asphyxia appeared to exhibit more convulsive (stage III–V) seizures than untreated controls (Table 2; Figure 3). This, however, was a random effect that was not observed when analyzing additional groups of untreated P11 rat pups (data not shown).

Mortality associated with asphyxia was low (Table 2) and not affected by litter size. Rat pups typically died during the last 5 minutes of asphyxia. The surviving rat pups exhibited no obvious alterations in body weight gain or general health status in subsequent weeks (not illustrated).

In view of the relatively short latencies between the end of asphyxia and onset of seizures, which is a potential problem for drug efficacy testing when drugs are administered after asphyxia (see below), we evaluated several modifications of the protocol illustrated in Figure 1. When the duration of intermittent asphyxia was increased from 30 minutes to 40 or 60 minutes, the average latency to convulsive seizures increased by only ~10% but mortality largely increased to 30% and 50%, respectively. We therefore decided to use the protocol illustrated in Figure 1 for drug experiments.

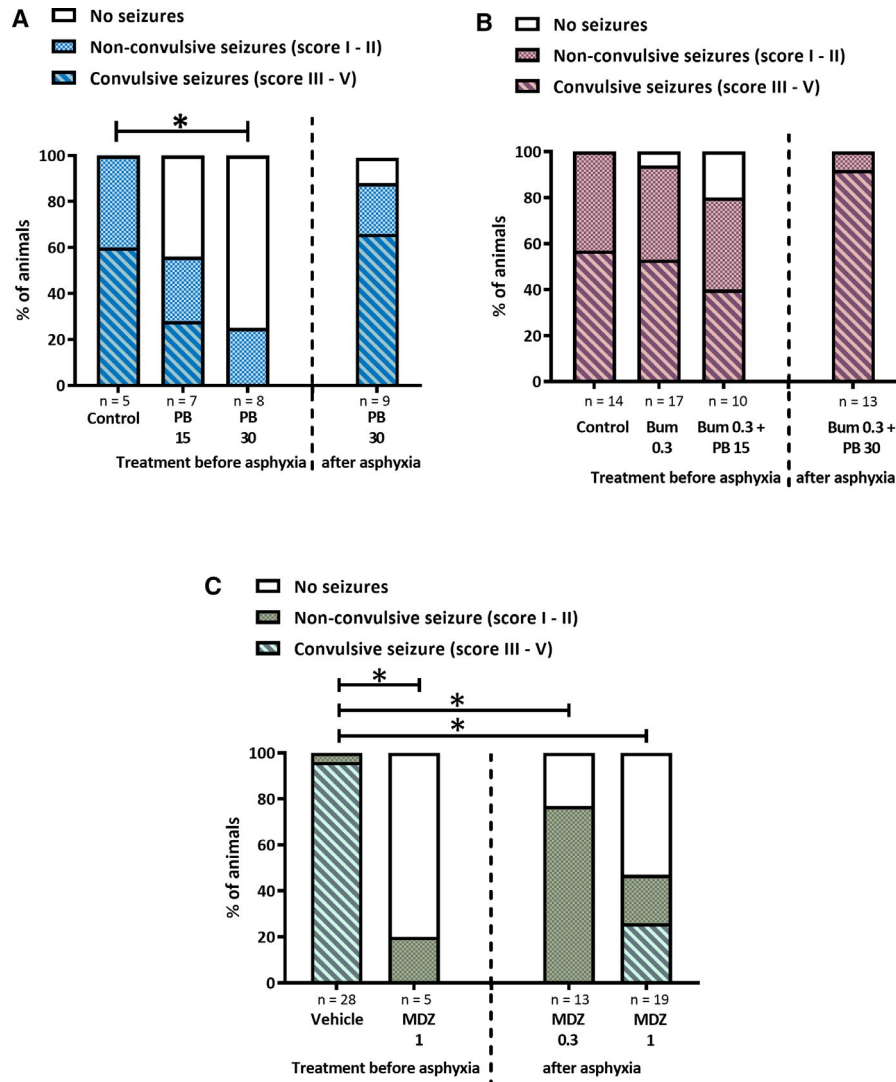


FIGURE 3 Drug effects on seizure incidence in the postnatal day 11 rat asphyxia model of neonatal seizures. Drugs or vehicle were administered either 15 or 30 minutes before onset of asphyxia or immediately after termination of asphyxia as shown in Figure 1. All types of seizures, including clinically obvious (stage I-V) seizures as well as other paroxysmal behavioral alterations and automatisms, were included in calculation of seizure incidence (see Tables 1 and 2); the highest seizure stage observed in each animal was used for the average values shown here. Seizure incidence is illustrated as percent of animals with seizures versus animals without seizures. Group size is indicated below the bars. Significant differences in seizure incidence from concurrent controls are indicated by asterisks ($P < .05$); actual P values are shown in Table 2. A, Effect of phenobarbital (PB). The drug was administered either at 15 and 30 mg/kg intraperitoneally (ip) 15 minutes before onset of asphyxia or at 30 mg/kg after termination of asphyxia. B, Effect of bumetanide (Bum) 0.3 mg/kg ip alone or in combination with 15 mg/kg PB on seizure incidence. Bum was injected 15 minutes and PB 30 minutes before onset of asphyxia. C, Effect of midazolam (MDZ). The drug was administered ip either at 1 mg/kg 15 minutes before onset of asphyxia or at 0.3 or 1 mg/kg immediately after termination of asphyxia. The MDZ vehicle was also either injected before and after asphyxia; because seizure incidence was 100% in both vehicle groups, they are shown together in one bar ($n = 28$)

3.2 | Effects of drug treatment

Treatment with 15 mg/kg phenobarbital 15 minutes before onset of asphyxia did not significantly reduce postasphyxic seizure incidence when compared to a concurrent untreated control group (Figure 3A). However, when compared to all untreated controls used in this study, a significant (50%) decrease in clinically obvious (stage I-V) seizures and a 38% decrease in all seizures were observed

in the phenobarbital-treated pups (Table 2). When the dose of phenobarbital was increased to 30 mg/kg, a significant 75% decrease in seizure incidence was obtained (Figure 3A, Table 2). The generalized convulsive (stage III-V) seizures were completely prevented by this dose, whereas nonconvulsive stage I-II seizures were resistant to treatment.

Bumetanide 0.3 mg/kg administered alone 15 minutes before onset of asphyxia did not significantly reduce seizures

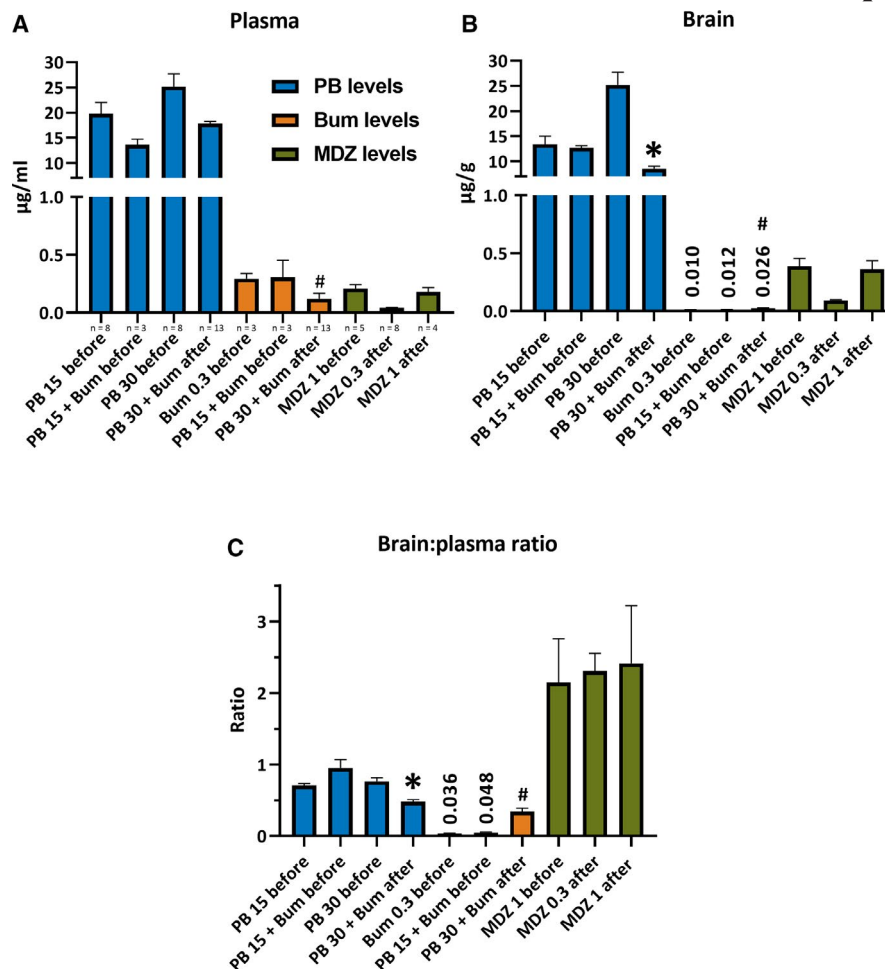


FIGURE 4 Plasma and brain levels of drugs in postnatal day 11 rat pups, determined 10 minutes after termination of asphyxia. As shown in Figure 1, drugs were administered either before or after asphyxia, which is indicated below the bars. Data are shown as mean \pm SEM; as shown below the bars in A, group size is 8 (phenobarbital [PB] 15 mg/kg before asphyxia), 3 (PB 15 mg/kg + bumetanide [Bum] 0.3 mg/kg before asphyxia), 8 (PB 30 mg/kg before asphyxia), 13 (PB 30 mg/kg + Bum 0.3 mg/kg after asphyxia), 3 (Bum 0.3 mg/kg before asphyxia), 5 (midazolam [MDZ] 1 mg/kg before asphyxia), 8 (MDZ 0.3 mg/kg after asphyxia), and 4 (MDZ 1 mg/kg after asphyxia), respectively. Significant differences in PB levels and brain:plasma ratio determined for 30 mg/kg administered either before or after asphyxia are indicated by asterisks ($P < .05$), whereas significant differences in Bum levels and brain:plasma ratio determined for 0.3 mg/kg administered either before or after asphyxia are indicated by hash signs ($P < .05$). With respect to these differences, please note that PB 30 mg/kg was either injected 15 minutes before asphyxia or immediately after asphyxia, and plasma and brain levels were determined in both experiments 10 minutes after asphyxia; this explains the lower brain levels when PB was administered after asphyxia, because PB penetrates into the brain only slowly.^{29,31} In contrast, when the same treatment protocol was used for Bum 0.3 mg/kg, Bum brain levels were higher when determined 10 versus 55 minutes after its injection, indicating rapid penetration into the brain but also rapid elimination as reported previously in adult rats.⁴² A, Plasma levels of PB, Bum, and of MDZ. B, Brain levels. The average brain level of Bum is shown above the respective bars. C, Brain:plasma ratio. The average brain:plasma ratio of Bum is shown above the respective bars

of any stage developing after asphyxia (Figure 3B; Table 2). Furthermore, a combination of phenobarbital (15 mg/kg; 30 minutes before asphyxia) and bumetanide (0.3 mg/kg; 15 minutes before asphyxia) did not exert any significant effect on seizures of any stage. Instead, combination with bumetanide tended to decrease the antiseizure efficacy of phenobarbital compared to treatment with phenobarbital alone ($P = .071$). Furthermore, in the group treated with bumetanide alone, 35% of the pups died compared to 15% in untreated controls, which, however, was not significant ($P = .1187$).

Obviously, ASDs can be administered to the neonate in the neonatal intensive care unit only after parturition when recovery from asphyxia starts, not during or before. Therefore, we performed experiments in which drugs were injected immediately after the termination of asphyxia (Figure 1). With phenobarbital 30 mg/kg, no significant antiseizure effect was observed. This can be explained by the short latent period (about 2 minutes; see above) between termination of asphyxia and onset of postasphyxic seizures, because phenobarbital penetrates into the brain and cerebrospinal fluid

relatively slowly.^{28,29} When bumetanide (0.3 mg/kg) was injected together with phenobarbital (30 mg/kg) shortly after asphyxia, again no significant antiseizure effect was determined (Figure 3B); however, the incidence of stereotypies was increased significantly (Table 2).

In contrast, midazolam (1 mg/kg) potently suppressed seizures when administered before or after asphyxia (Table 2; Figure 3C). When administered before asphyxia, the antiseizure effect of midazolam was similar to that of high-dose phenobarbital in that a significant 80% decrease in seizure incidence was observed. Again, the generalized convulsive (stage III-V) seizures were completely prevented by this dose of midazolam, whereas nonconvulsive stage I-II seizures were resistant to treatment. When midazolam (1 mg/kg) was injected after asphyxia, seizure incidence was reduced by 53%. Benzodiazepines are known to penetrate into the brain within seconds after parental injection,³⁰ thus explaining the beneficial efficacy compared to phenobarbital when injected after asphyxia in our model.

Based on the significant antiseizure effect of midazolam (1 mg/kg) injected after asphyxia, we performed an additional experiment in which we decreased the dosage of midazolam to 0.3 mg/kg. As shown in Table 2 and Figure 3C, midazolam significantly reduced the incidence of seizures by 33%, thus demonstrating that the effect of midazolam was dose-dependent. However, the generalized convulsive (stage III-V) seizures were completely prevented by this dose of midazolam, whereas nonconvulsive stage I-II seizures were resistant to treatment.

In none of the drug experiments were obvious differences between male and female pups observed (Figure S1). At the lower dose of phenobarbital, male rat pups appeared to be more responsive to the anticonvulsant effect of this drug than female pups; however, the low sample size per sex excluded any meaningful quantification. Only for the experiments with the higher dose of midazolam (1 mg/kg) after asphyxia was group size per sex large enough for determining potential sex differences. The number of midazolam responders was similar in male (6/9) and female (5/10) rat pups in this experiment (Figure S1C).

In addition to determining the effects of drugs on seizure incidence, we also evaluated whether drug treatment exerted an effect on seizure latency. As shown in Table S2, except for midazolam (1 mg/kg after asphyxia), none of the treatments significantly altered the latency to seizure onset after asphyxia. Midazolam increased the latency to nonconvulsive seizures 2.4-fold compared to seizure latency determined after injection of the midazolam vehicle.

All drug experiments described above were performed with ip administration, whereas the clinical route of treatment of neonatal seizures is typically intravenous (iv).³ Preliminary experiments in P11 rat pups showed that iv administration via the tail veins is easily possible before asphyxia. However,

immediately after asphyxia, the tail veins were constricted, most likely as a consequence of circulatory centralization, so that iv injection was successful in only ~20% of the pups. We therefore decided to skip the iv route in the present study.

3.3 | Drug levels in plasma and brain

When phenobarbital (15 or 30 mg/kg ip) was administered 15 minutes before onset of asphyxia and plasma and brain drug levels were determined 10 minutes after termination of asphyxia, that is, shortly after the postasphyxic seizures, brain levels of phenobarbital were only moderately lower than those determined in plasma, with average brain:plasma ratios of 0.71 (15 mg/kg) and 0.77 (30 mg/kg), respectively (Figure 4). Combined treatment with bumetanide did not significantly affect plasma or brain levels of phenobarbital (15 mg/kg) or its plasma:brain ratio (Figure 4). When phenobarbital (30 mg/kg) was administered immediately after asphyxia and plasma and brain levels were determined 10 minutes later, brain levels and the brain:plasma ratio of phenobarbital were significantly lower compared to the values determined 55 minutes after drug administration (Figure 4B,C). This can be readily explained by the relatively slow brain penetration of this drug, which reaches maximal brain levels only at ≥ 2 hours after iv administration in rats.³¹

In contrast to phenobarbital, as reported previously for adult rodents,^{24,32,33} bumetanide only poorly penetrated into the brain, resulting in an average brain:plasma ratio of only 0.036, when bumetanide (0.3 mg/kg) was injected 15 minutes before asphyxia (Figure 4C). Significantly higher brain levels were determined when the same dose of bumetanide was injected immediately after asphyxia (ie, about 10 minutes before determining plasma and brain levels; Figure 4B), thus indicating a very rapid (but incomplete) penetration of this drug into the brain. Phenobarbital did not alter the plasma or brain levels of bumetanide (Figure 4). Thus, as shown previously,¹⁴ the blood-brain barrier (BBB) of neonatal rats is not more penetrable by bumetanide than the BBB of adults, so that brain bumetanide levels (~ 0.01 – 0.03 $\mu\text{g/mL}$ = 0.028 – 0.084 μM) were far below those needed to inhibit NKCC1, particularly when considering the extensive brain tissue binding of bumetanide (cf Brandt et al³³).

It is also important to note that in rodents, bumetanide is much more rapidly eliminated than phenobarbital. The average elimination half-life of bumetanide in adult rats is only ~ 10 minutes²⁴ compared to 9–20 hours for phenobarbital³⁴; the interdrug difference in elimination half-life may be even higher in neonatal rats.

Midazolam (1 mg/kg) achieved about the same plasma and brain concentrations, irrespective of whether the drug was administered before or after asphyxia (Figure 4). Brain:plasma ratio was about 2.2–2.4, indicating brain accumulation of this

lipophilic drug. At the lower dose of midazolam (0.3 mg/kg), administered after asphyxia, plasma and brain levels were about one-third of those determined at 1 mg/kg, but the brain:plasma ratio was the same (Figure 4).

4 | DISCUSSION

The present study illustrates the antiseizure efficacy of clinically used ASDs in a recently described novel rat model of birth asphyxia,¹⁶ whereas the NKCC1 inhibitor bumetanide is ineffective. To our knowledge, this is the first rodent model of postasphyxial neonatal seizures that allows testing of drugs after asphyxia, because, as in human neonates, seizures occur during establishment of normocapnic conditions (see Introduction). As shown here, administration of midazolam after asphyxia blocked seizures in about 50% of the rat pups, whereas phenobarbital was not effective, most likely as a consequence of its slow brain penetration. When high doses of phenobarbital or midazolam were administered shortly before asphyxia, both drugs blocked postasphyxial convulsive seizures, whereas nonconvulsive seizures were resistant. Bumetanide was not capable of potentiating the effect of phenobarbital, independently of whether it was administered before or after asphyxia. The present translational data obtained with the novel rat model of birth asphyxia indicate that this model is a useful tool to evaluate novel treatments for neonatal seizures.

As discussed in detail recently,^{15,16} the present model of asphyxia-induced neonatal seizures in P11 rat pups parallels human birth asphyxia but differs in several important aspects from the pure-hypoxia models that are widely used in neonatal research.³⁵ As pointed out in the Introduction, in human neonates, the most common cause of postnatal seizures is prolonged perinatal asphyxia, exposing the fetus or newborn to hypoxia and hypercapnia with significant acidosis, which suppresses neuronal activity during asphyxia. This is also observed in the present model,¹⁶ as exemplified by the decreased EEG amplitude during asphyxia. As in human neonates, seizures are observed during recovery from asphyxia, and these seizures are paralleled by a rise in blood and brain pH.^{15,16,36} The postasphyxial seizures observed in the present model cover the whole spectrum of neonatal seizures that occur in human newborns.¹ Thus, the present model closes an important gap (see, eg, Lombroso³⁷) between the laboratory and the clinic. In line with this conclusion, the pharmacology of the postasphyxial seizures in P11 rat pups seems to be more translationally relevant than previous drug studies in other models of neonatal seizures, in which the seizures were induced by hypoxia or the excitotoxic convulsant kainate.^{14,38–40}

The negative outcome of the present experiment with bumetanide (0.3 mg/kg ip) as adjunct to phenobarbital (15

or 30 mg/kg ip) for treatment of postasphyxial neonatal seizures is in line with the negative outcome of the European multicenter NEMO trial reported by Pressler et al.¹³ Thus, the present asphyxia model correctly predicts the failure of bumetanide to increase the antiseizure efficacy of phenobarbital in human neonates. This is in contrast to the study performed by Cleary et al.¹⁴ in a noninvasive rat hypoxia model, in which bumetanide (administered before hypoxia) potentiated the antiseizure efficacy of phenobarbital with exactly the same doses and pretreatment times that have been used here. Interestingly, in a mouse model of ischemia-induced neonatal seizures in mice, bumetanide (0.1–0.2 mg/kg) also failed to potentiate the action of phenobarbital (25 mg/kg) at P7 and P12, and significantly blunted the effect of phenobarbital at P10.⁴¹ Thus, when using an etiologically relevant rodent model of neonatal seizures, bumetanide, administered at clinically relevant doses, is not an effective adjunct of phenobarbital.

This is not surprising, because bumetanide only poorly penetrates into the brain and is highly bound to brain lipids and proteins, so that it does not reach NKCC1-inhibitory brain levels at clinically relevant doses.³³ Thus, it is difficult to explain by which mechanism(s) clinically used doses of bumetanide (0.1–0.5 mg/kg ip) potentiated the antiseizure effect of phenobarbital¹⁴ or suppressed seizures when administered alone⁴⁰ in hypoxia models of neonatal seizures. Effects of bumetanide on apically expressed NKCC1 in the BBB, off-target effects (not mediated by NKCC1), and systemic effects of bumetanide have been discussed in this context.^{9,12,33} At a much higher dose (10 mg/kg ip), bumetanide enhanced the antiseizure effect of phenobarbital in an invasive neonatal cerebral hypoxia-ischemia rat model, but this effect was lost at 2.5 mg/kg bumetanide.³⁹ At 10 mg/kg, unbound brain levels of bumetanide reach the concentration range (0.1–0.3 μ M) needed to inhibit NKCC1.³³ It will be interesting to see whether the more BBB-permeable bumetanide prodrugs examined in our previous work⁴² are more efficient than the parent compound in the present model.

In contrast to the inability of bumetanide to potentiate the antiseizure effect of phenobarbital (15 mg/kg) in the present model, the higher, still clinically relevant dose of phenobarbital (30 mg/kg) was quite effective to reduce seizure incidence when applied before, but not after, the asphyxia exposure. Similarly, the potent benzodiazepine midazolam suppressed the seizures, which is in line with its use as a second-line ASD for neonatal seizures.⁵ Interestingly, midazolam was effective in suppressing the seizures when injected either before or after asphyxia (before seizure onset), thus substantiating its rapid brain penetration. It may be argued that the effective dose (1 mg/kg) of midazolam used here was considerably higher than the average dose (0.15–0.3 mg/kg) used clinically.⁴³ However, as a result of different administration routes (ip here and iv clinically) and species differences

in drug elimination and body surface area,⁴⁴ the average plasma levels 10 minutes after ip administration of midazolam (1 mg/kg) in rat pups (~0.18 µg/mL) were in the range (~0.14–0.2 µg/mL) determined 10 minutes after iv administration of 0.1–0.2 mg/kg in pediatric patients.^{45,46} At a lower, clinically relevant dose of midazolam (0.3 mg/kg), which was administered after asphyxia, seizures were only prevented in ~30% of the rat pups. However, the severe generalized convulsive (stage III–V) seizures were completely prevented by this dose of midazolam.

Despite the efficacy of midazolam and phenobarbital to block convulsive neonatal seizures, both drugs were ineffective to suppress nonconvulsive seizures, that is, the most frequent type of neonatal seizures in humans.⁵ Furthermore, both drugs may induce apoptotic neuronal death in specific regions of the immature brain⁴ and increase neonatal seizure-associated neuronal injury,⁴⁷ so novel treatments for neonatal seizures are urgently needed.⁴⁸ The present model of asphyxia-induced neonatal seizures may help to identify such novel therapies.

Some of the properties of the present model of birth asphyxia have immediate implications for the technical design of pharmacological experiments. The onset of epileptiform activity is fast, starting around 2 minutes after the end of asphyxia. Although this is a clear advantage over noninvasive hypoxia-only models, in which seizures occur during hypoxia,¹⁶ the short latency between asphyxia and onset of seizures in the present model complicates drug testing for pharmacokinetic reasons. This limits the range of ASDs that can be tested for antiseizure efficacy after asphyxia to drugs such as midazolam, which penetrates extremely fast into the brain,³⁰ whereas drugs such as phenobarbital do not reach sufficiently high levels at their brain targets within the time frame necessary. This is illustrated by the different efficacy of high-dose phenobarbital administered before versus after asphyxia in the present study. Bumetanide did not potentiate phenobarbital when being administered as adjunct to phenobarbital after asphyxia. A study design with drug administration after asphyxia would better represent the clinical treatment of human birth asphyxia.

Interestingly, in the experiments of Kang et al,⁴¹ in which phenobarbital (25 mg/kg ip) was given 1 hour after onset of permanent unilateral carotid-ligation to produce acute ischemic seizures on P7, P10, and P12 CD1 mice, phenobarbital was effective as an antiseizure agent at P10 and P12, but bumetanide as an adjunct failed to improve the efficacy of phenobarbital. Thus, the outcome of our study design with administration of phenobarbital and bumetanide before and after asphyxia is similar to the results of Kang et al⁴¹ with administration of these drugs after onset of an ischemic insult.

In human neonates, ASDs are typically administered iv, whereas we used ip administration here, which has pharmacokinetic limitations such as lower bioavailability and slower

onset of pharmacological effects compared to the iv route. As described in Results, we tried to inject drugs iv before and after asphyxia in rat pups, but the yield of successful iv drug injections after asphyxia was too low to allow using this route routinely in large groups of pups. This was a result of circulatory centralization after asphyxia, leading to tail vein constriction, which is inherent in all valid noninvasive models of asphyxia. Chronic implantation of iv catheters before onset of asphyxia and some of the iv injection techniques that have been described previously in neonatal rodents^{49–52} are feasible alternatives for future experiments, although all these techniques are exacting procedures.

Increased neuronal NKCC1 expression has been implicated in neonatal seizures in humans^{6,9–12} (although data supporting this hypothesis are missing), but the role of NKCC1 upregulation, if any, in the present model is not yet known. Cleary et al¹⁴ reported that the expression of NKCC1 protein transiently increased in cortex and hippocampus after hypoxic seizures in P10 rat pups. In apparent contrast, no significant changes were detected in NKCC1 expression in the mouse model of ischemia-induced neonatal seizures used by Kang et al.⁴¹ We plan to quantify NKCC1 brain expression in our model as well, using novel specific antibodies together with proper NKCC1 knockout controls for the visualization of endogenous NKCC1 in distinct cell types and their subcellular compartments. In this respect, it is important to note that (1) most of the NKCC1 in brain tissue is expressed in glial cells, oligodendrocytes, astroglia, and microglia but not in neurons; and (2) the lack of specific NKCC1 antibodies has thus far complicated the interpretation of immunochemical studies on the subcellular distribution of NKCC1.^{8,9} These technical issues seem to explain most of the discrepancies in NKCC1 expression in the pertinent literature.

In conclusion, as outlined in detail recently,^{15,16} the non-invasive rat model used here is the first physiologically-valid rodent model of birth asphyxia with neonatal seizures triggered after the insult. The present pharmacological data indicate that this model is a useful tool to evaluate current as well as novel treatments for neonatal seizures. Furthermore, our model may be used to study the detrimental long-term consequences of neonatal asphyxia and seizures and whether it is possible to prevent or minimize such consequences by pharmacological intervention after asphyxia.

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CONFLICT OF INTEREST

None of the other authors has any conflict of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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